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Characterization of polygalacturonases produced by the endophytic fungus *Penicillium brevicompactum* in solid state fermentation - SSF

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***Abstract*—** *Polygalacturonases belong to the family of pectinases, enzymes that are in high demand in industry because of their many different applications. This study therefore sought to examine the production of polygalacturonases using an endophytic fungus, Penicilium* brevicompactum, *isolated from Baccharis dracunculifolia D.C. (Asteraceae) through semi solid fermentation using orange peels and citric pectin 2% as base substrate, supplemented with different carbon sources. After the fermentation process, the enzyme was characterized. The results showed that the micro-organism was able to use a wide range of carbon sources, but with polygalacturonase activity varying with each source. The highest yield, however, was achieved after 30 hours of incubation in the presence of 4% of galactose and 2% of pectin. Studies on the characterization of the polygalacturonase revealed that the optimal temperature of this enzyme is 72°C and that it maintains 60 and 15% of its maximum activity when incubated for 2 hours at 40 and 90°C, respectively. The optimal pH for the activity of the enzyme was 4.6. The enzyme retained 65 and 30% of its maximum activity when incubated at pH 3.5 and 9.5, respectively, for 24 hours at ambient temperature. The enzyme activity was stimulated by Mg^{2+} ions. On the other hand, it was inhibited by the ions Cs^{+2}, Hg^{+2}, Li^{+2} and Sr^{+2}. The ions Zn^{+2} and Cu^{+2} inhibited it by 94% and 69%, respectively.*

***Keywords*— Enzymes, pectins, fermentation, polygalacturonase, bioprocess.**

I. INTRODUCTION

Pectins are classes of complex polysaccharides found in the cell walls of plants and they are commonly produced during the early growth stages of the cell wall, corresponding to approximately 1/3 of this wall (Sajjaanatakul; Van-Buren and Downing, 1989; Muralikrishna and Taranathan,1994).

The synthesis of pectin (Figure 1) starts with the UDP-d-galacturonic acid and it is synthesized through the Golgi Complex during the early growth stages of the cell wall. Although galacturonic acid is the main constituent of pectic substances, varying proportions of other sugars, such as D-galactose, L-arabinose, D-xylose, L-rhamnose, L-fucose and traces of 2-O-methyl fucose can also be found (Jarvis, 1984; Leitão *et al.*, 1995).

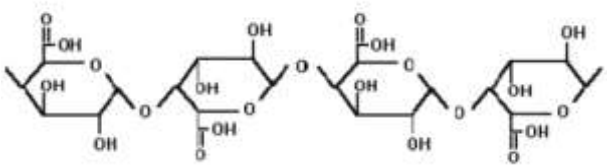

Fig. 1: Chemical Structure of pectin, formed by sub-units of galacturonic acid (Houdert and Muller, 1991).

It has been suggested that the carboxyl groups of pectins are strongly methyl-esterified, but that these esters are cleaved by the PME (pectin methylesterase) present and by the activity of polygalacturonase in tomatoes, resulting in pectins of high molecular weight. Roberts, (1990), Tieman and Handa (1994), described that the decreased activity of PME in tomatoes causes a total loss of integrity of tissues during the senescence of the fruit. PME accelerates the hydrolysis of the ester-methyl bonds in the pectin molecule, forming pectic acid and methanol (Amstalden, 1982).

Although several enzymes called hydrolases of the pectinase type are responsible for the degradation or breaking of this polymer contained in the plant's cell wall, the activity of pectin methylesterase (PME) and polygalacturonase (PG) stands out, which act during the

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ripening of the fruit and which have been extensively investigated regarding their genetics, biochemistry and levels of gene expression (Fischer; Bennett, 1991).

In Kashyap *et al.*, (2001) and Belafi-Bako *et al.*, (2007), pectinolytic enzymes (Figure 2) are classified according to their mode of action as demethylation pectins, which catalyze the hidrolise of methoxyl groups linked to carboxylic groups of the pectin chain, forming pectic acid, which contains negligible quantities of methoxyl groups (pectinesterase (PE): E.C. 3.1.1.11); depolymerizing pectins, which catalyze hydrolysis reactions (polymethyl-galacturonases (PMG), polygalacturonases (PG): endo-PG (E.C. 3.2.1.15) and exo-PG 1 and 2 (E.C.3.2.1.67, E. C. 3.2.1.82)) and the break up by trans-elimination (polygalacturonatelyase (PGL): endo-PGL (E. C.4.2.2.2) and exo-PGL (E. C.4.2.2.9) and pectin lyase (PL) (4.2.2.10) (Sakai and Okushima, 1982; Ahrens and Huber, 1990; Fischer and Bennett, 1991; Kashyap *et al*., 2001; Belafi-Bako *et al.*, 2007).

Pectinolytic enzymes can be synthesized by bacteria, fungi and yeasts. The production of pectinases by micro-organisms is influenced by growing conditions, and especially by the composition of the culture medium, the type and concentration of the carbon source, pH and temperature of cultivation, in addition to other factors (Fogarty and Ward, 1974; Freitas, 1991; Bravo *et al.*, 2000).

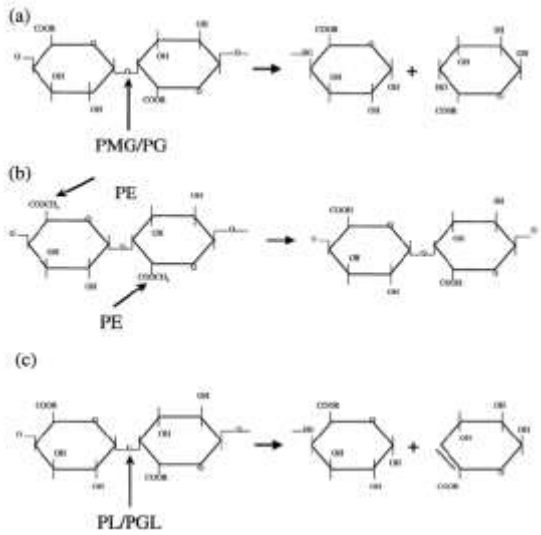

Fig. 2: Different types of pectinases and their modes of action on pectic substances. (a) R = H for PG and CH3 for PMG, (b) PE and (c) R = H for PGL and CH3 for PL. The arrow indicates the place where the pectinases act on the pectic substances. PG: polygalacturonase, PMG: polymetylgalacturonase, PE: pectinesterase, PGL: pectate lyase, PL: pectin lyase (Gummadi and Panda, 2003).

The first step for the industrial use of these enzymes is the selection or development of appropriate strains, followed by an optimization of the cultivation conditions. In other words, one has to learn the various aspects that regulate the synthesis and activity of the enzyme. The micro-organism is, without doubt, the limiting factor of any fermentation process. This fact is commonly corroborated in the literature, as for example in (Maiorano, 1982; Maiorano and Schmidell; Ogaki, 1995; Nogueira, 2003; Collares, 2011).

These enzymes can be obtained through both submerged (SmF) and solid state (SSF) fermentation processes (Fogarty and Ward, 1974; Freitas, 1991; Bravo *et al.*, 2000; Gummadi and Panda, 2003; Collares, 2011). For the specific case of SSF, the supports or substrates consist basically of organic polymers that are characterized by their insolubility in water and their ability to promote microbial growth, even without the addition of additional nutrients. Among the main characteristics of SSF, the following deserve special mention: low quantity of available water, low risk of contamination, use of smaller reactors than those used in SmF for the same amount of substrate, high yields and low costs with respect to the substrate used (Hendges, 2006).

The production of enzymes by micro-organisms through SSF is influenced by several cultivation factors, such as: the micro-organism used in the fermentation process, the availability of water, nutrients, temperature, the applied inoculum, aeration and the presence of inducing substances. The study of these factors is essential for the optimization of a fermentation process (Dartora *et al.*, 2002).

Pectinolytic enzymes are widely employed in various industries. In food, these enzymes are used for the extraction, depectinization and clarification of fruit juices (bananas, papayas, apples) and wine, for the extraction of vegetable oil, and in the production of baby foods (Buenrostro and Lopes-Munguia, 1986; Ghildyal *et al.*, 1994; Kashyap *et al.*, 2001; Nighojkar *et al.*, 2006; Gupta *et al.*, 2007; Ustok, Tari and Gogus, 2007). They can also be used in the industry of fermented products, such as the fermentation of cocoa, coffee and tobacco, and in the degumming of natural fibers (Genari, 1999; Bravo *et al.*, 2000; Fawole and Odunfa, 2003).

The food industry, juice producers in particular, has shown a growing interest in the use of pectinases since their use in various processes results in increased yields, quality improvements of the product and reductions in the operating costs of the process (Wosiacki and Nogueira, 2005). Micro-organisms are a rich source of many enzymes, and pectinases, particularly those produced by fungi, are of great industrial importance (Koch; Nevins, 1989; Ferreira and Castilho; Paiva, 1995; Uenojo and Pastore, 2007).

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It's in this context that this study observed the production of fungal polygalacturonases by the endophitic fungus *Penicillium brevicompactum*, isolated from *Baccharis dracunculifolia* D.C. (Asteraceae), using Solid State Fermentation (SSF) in a medium made up of dehydrated orange peels, inductor sources and salts.

II. MATERIALS AND METHODS

2.1 Microrganisms

The strain of *Penicilium brevicompactum* used in this work was isolated by Recalcatti *et al*., (2004). The methodology used for isolation has been described by Day, Fincham and Radford, (1979) and Larone, (1985). The conservation of this strain was done in a glycerinated culture medium, following the methodology described by Maiorano, (1982), being maintained through monthly samplings.

2.2 Culture conditions

The culture medium was formulated containing ($g.L^{-1}$) $MgSO_4.7H_2O$ 0.2g, $(NH_4)2SO_4$ 0.2g, KH_2PO_4 0.05 g. This formulation of salts was supplemented with the following carbon sources: citric pectin 2g; galactose 2g, galactose 4g and polygalacturonic acid 4 g in 100 g of orange peels, dried until reaching a humidity of 10%, crushed and sieved to remove particles of smaller size and obtaining fragments with more homogeneous dimensions of approximately 3 mm (Gomes, 1995).

The pH value of the medium was adjusted to 4.5 and the sterilization was performed for 15 min at 120 ºC. The vials, properly capped with cotton stoppers, were sterilized at 120 ºC for 15 minutes and the pressure was maintained at 0.5 atm to avoid caramelization of the culture medium.

Considering the conditions established in the preliminary fermentation, the kinetics of enzyme production were monitored for a period of 96 hours at 35 ºC, taking samples after 0, 20, 40, 60, 80, 100 and 120 hours.

2.3 Enzymatic assay of polygalacturonase

The fermented mass, collected at certain periods of cultivation, was homogenized and macerated (wet) in a mortar. Samples were collected after 0, 20, 40, 60, 80, 100 and 120 hours.

To determine the moisture content, 1g of the sample was submitted to oven drying at 105 °C until reaching a constant weight. After cooling, the sample was weighed again and the moisture content present in the medium was calculated, (AOAC, 1997).

To determine the pH, 10 mL of distilled water was added to the dried samples used for the determination of moisture. After shaking, these were allowed to rest for 10 min, and then the pH reading was performed. The biomass was determined after filtration and the micelles were submitted to heating at 85 ºC until reaching a constant weight.

For the extraction of enzymes, 15 mL of destilled water at pH 4.0 was added to 2.7 g of the previously macerated fermented mass. After 30 min of shaking at 200 rpm, the suspension was centrifuged at 9000 rpm for 20 min. The broth (15 mL) was then used to determine enzyme activity.

Polygalacturonase activity was determined in the filtered cultures of *P. brevicompactum* by determining the content of reducing groups, after reaction with dinitrosalicylic acid (Miller, 1959). The mixture of the reaction contained: 2.4 mL of citrate/phosphate buffer 0.2M, pH 5.0, 0.1 mL of enzyme extract and 2.5 mL of partially methoxylated citric pectin, 0.25% (Sigma, P-9135). The pre-incubation was carried out at 35°C for 10 minutes.

The reaction was initiated by the addition of enzyme extracts and water was added in the control test instead of the substrate (Malvessi, 2000; Malvessi and Silveira, 2004). One unit of activity was considered to be the amount of enzyme capable of generating 1 mol of galacturonic acid, per minute.The content of free reducing sugars (RS) present in the enzymatic extract was determined by the DNS method, using a glucose solution as standard.

To determine the total reducing sugars (TRS) in the fermentation medium, the methodology described by Malvessi, (2000), was used; Malvessi and Silveira, (2004), in which 10 mL of H_2SO_4 1.5 M was added to 0.7 g of homogenized and macerated mass, hydrolyzed at 100°C for 30 min, followed by cooling in an ice bath. After the neutralization, the preparations were deproteinated and filtered, and the reducing sugars obtained through this technique were then quantified through the DNS method (Miller, 1959).

2.4 Characterization of the enzyme

The equivalent of 60% of ammonium sulfate was added to the cell-free supernatant for protein precipitation. After this procedure, the material precipitate was centrifuged at 20.000 revolutions for 10 minutes at 4°C. A minimum quantity of phosphate buffer was added to the precipitate for its re-suspension, after which it was once again centrifuged at 20.000 revolutions per 20 minutes at 4°C. The filtrate was used for determination of the enzyme activity (Malvessi and Silveira, 2004).

2.5 Effect of temperature on the activity and stability of polygalacturonase

The determination of optimal temperature was performed by incubating the reaction mixture (pH 7.0) at temperatures ranging from 40 to 100°C, with intervals of 10°C. After 10 minutes of incubation at each temperature, the enzyme activity was analyzed. The thermal stability was evaluated by incubating the enzyme in temperatures that ranged from 30 to 100°C, with intervals of 10°C. After two hours of

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incubation, the residual activity was examined at the optimal temperature of the enzyme determined above.

2.6 Effect of the pH on the activity and stability of polygalacturonase

The influence of pH on the activity of polygalacturonase was evaluated in the range of 3.5 to 9.5 with an interval of 0.5 units. The substrate preparation used was a buffering mixture containing sodium acetate (pH 5.0 -5.5), phosphate (pH 6.0 - 8.0) and tris (pH 8.5 -10.0), with a final concentration of 50 mM. The pH values of the reaction mixture were adjusted with NaOH or HCl 1N.

The optimal pH was determined by incubation of 0.2 mL of the enzyme and 0.8 mL of citric pectin (0.5%) prepared in buffers with different pH values. After incubation at 70°C for 10 minutes, the enzyme activity was analyzed as described previously. The stability of the polygalacturonase under different pH values was evaluated by incubating the enzyme extract in the buffers described above, without the substrate, for 24 hours at ambient temperature. After this treatment, the residual activity of the polygalacturonase was determined as described previously.

2.7 Effect of ions on the activity and stability of polygalacturonase

The following compounds were added to the reaction mixture, at a final concentration of 1 mM, to study the effect of different metal ions on the activity of polygalacturonase: $CaCl_2$, $BaCl_2$, $AgNO_3$, $HgCl_2$, $CuSO_4$, $ZnSO_4$, CsCl, $CoSO_4$, $NiCl_2$, $C_4H_6O_4Pb$, $FeSO_4$, $MnSO_4$, $MgSO_4$, $SrCl_2$and $LiSO_4$. This method followed that described by Malvessi (2000) and Malvessi and Silveira (2004).

2.8 Statistical analysis

The experiments were carried out in triplicate, and the results were evaluated by analysis of variance (ANOVA) using the SAS software, version 9.4. The effects of the treatments were compared with Tukey's Test.

III. RESULTS AND DISCUSSION

The carbon source in the growth medium of the fungi interferes with the synthesis of extracellular polygalacturonase. The increase in the synthesis of polygalacturonase by adding pectin (Maldonado and Calieri, 1989; Larios, Garcia and Huitron, 1989; Bailey, 1990; Baracat *et al.*, 1991), or galactose (Polizeli, Jorge and Terenzi, 1991), to the growth medium, has been observed with other micro-organisms.

This study was performed using galactose and polygalacturonic acid on pectin 2% as carbon sources in order to analyze the biomass, protein and polygalacturonase activity of *P. brevicompactum*. The highest production of mycelial biomass (6.34 mg/g), polygalacturonase activity (15.25 U/g) and total reducing sugars - TRC (0.84 g/g), was observed in the medium containing 4% galactose and 2% of citric pectin at pH 6.9 (Table 1).

Table.1: Effect of the nature of the carbon source on the accumulation of final biomass, total protein, activity and productivity of polygalacturonase in Penicillium brevicompactum. Time of 60 hours of fermentation.

Carbon[1]	Biomass	PG Activity[2]	TRS[3]	pH	Moisture
A	5.22a*	9.12b	0.33b	4.5a	50a
B	5,56a	8.20b	0.23b	4.6a	60a
S	6,34a	15.25a	0.84a	4.6a	55a
D	5,25a	10.21b	0.35b	4.4a	60a

[1]A= Pectin 2%. B= Pectin 2% + Galactose 2%. C= Pectin 2% + Galactose 4%. D= Pectin 2% + Polygalacturonic Acid * 4%. [2] Polygalacturonase Activity. [3]TRS - Total Reducing Sugars. * Means followed by the same letter vertically, do not differ by Tukey's Test (< 0.05).

In all tests, a drop in pH could be observed during the first 6 hours of incubation of the culture. A decrease in pH from 4.5 to 4.3 of the culture medium could be detected.

This initial drop in the pH of the medium may be due to the production of acids during the fermentation of the carbon source, which is more intense during this period. Subsequently, the pH of the medium increased gradually to 6.8 after 40 hours of incubation of the culture, remaining virtually unchanged until 60 hours, after which it decreased, maintaining itself stable at 4.6 for the medium containing pectin 2% + Galactose 4%. There was no significant difference in the mean pH values of all evaluated media. The increase in pH of the medium may be attributed to the use of organic acids or the production of alkali components during this period.

According to Ming Chu, Lee and Li, (1992), the acidification or alkalinization of the culture medium reflects the consumption of the substrate. When ammonium ions are being used, the environment becomes more acid, and when organic nitrogen (amino acids and peptides) is being assimilated, the environment becomes more alkaline. Due to this relationship between the synthesis of polygalacturonase and the use of nitrogenous compounds, the variation of pH can be used to provide important information about the production of polygalacturonases, such as the beginning and the end of its synthesis.

The effect of incubation time in the polygalacturonase trial was tested in a series of experiments in which the incubation time of the reaction at 30°C varied in the range of 20 to 120 minutes. The activity of the polygalacturonase

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is at its maximum at 30 minutes of incubation of the reaction (Figure 3).

After 30 minutes of incubation of the reaction, the concentrations of reducing substances decrease as time increases, which means an up to 3-fold drop in the polygalacturonase activity in the fermentation supernatant from the fungus *P. brevicompactum*, with 60 minutes of incubation.

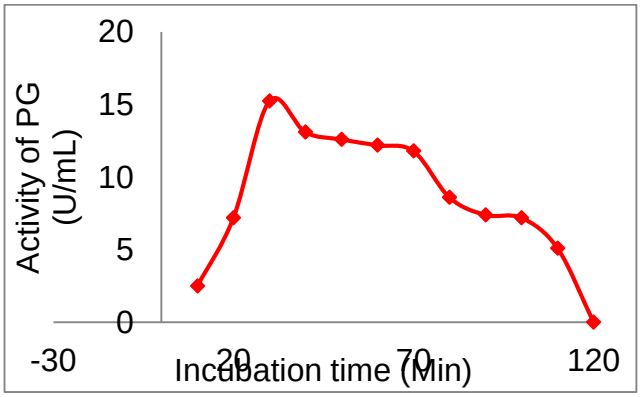

Fig.3: Production of polygalacturonase as a function of time, for Penicillium brevicompactum.

The results obtained in this work show that enzyme activity increased with the addition of galactose to 4% associated with the citric pectin at 2%. This same behavior was observed by Bueno, Peres and Gattas, (2005) who evaluated the behavior of different strains of *Aspergillus* sp. and found that the best results occurred with the association of citric pectin and galactose.

Freitas, (1991) showed that increases in the concentration of citric pectin didn't result in a considerable increase of enzyme activity, showing that the production of polygalacturonase by *Penicillium expansum* initially increased with the rising concentration of pectin in the medium, after which it decreased, because although the fungus continues growing, the polygalacturonase activity varies in response to the carbon source used for the growth of the micro-organism. The activity of the polygalacturonase was significantly higher during the growth of the micro-organism, changing the carbon source, associated with citric pectin.

3.1 Effect of temperature

Polygalacturonase activity increased with the increase in temperature, reaching its maximum value at 72°C, as shown in Figure 4.

The polygalacturonase secreted by *P. brevicompactum* maintained around 72% of its activity when incubated at 70°C for 2 hours and reduced its stability with the increase in temperature. It should be emphasized that this stability with temperatures near 70°C is a good indicator, as thermostable enzymes have a good market acceptance.

Martins *et al.*, (2002) obtained polygalacturonase from *Thermoascus aurantiacus* that retained 100% of its activity when incubated at 60 °C for 2 hours. A 50% reduction in the activity of this enzyme was observed, however, after incubation at 70 °C for 2 hours. These results indicate the production of thermostable polygalacturonase.

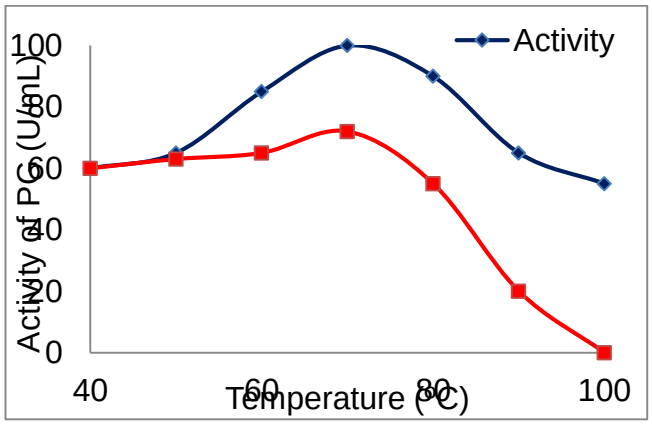

Fig. 4: Optimal temperature and thermal stability of polygalacturonase secreted by *Penicillium brevicompactum.*

3.2 Effect of pH

The polygalacturonase was active in a wide pH range, with maximum activity around pH 4.6, as shown in Figure 5. The lowest enzyme activity was found at the pH values of 3.5 and 9.5, which were the most extreme values studied, with 65 and 40 %, respectively.

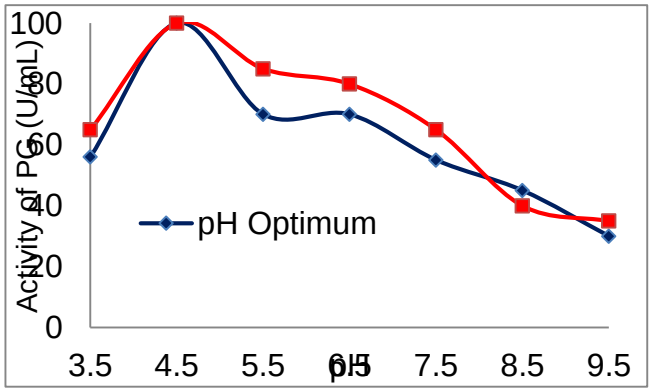

Fig.5: *Influence of pH on the activity and stability of the polygalacturonase secreted by* Penicilium brevicompactum.

Regarding the stability of the pH, the polygalacturonase was stable for 24 hours at pH values between 4.5 and 5.0. When incubated at pH 9.5, the enzyme stability was only 30%.

Similar results were found by Rizatto (1999); Alaña, Llama and Serra, (1991), and Silva *et al*., (2005), who investigated the effect of pH on the polygalacturonase activity of *Penicillium italicum* and *Aspergillus niger* through semi-solid fermentation in industrialized orange bagasse.

According to these authors, these filamentous fungi studied revealed an optimal pH for polygalacturonase activity

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between 4.5 and 5.5, thus producing acid enzymes. Different Results are observed for cultures with different *Bacillus* strains, which produce enzymes in an optimal pH between 6.5 and 7.0. Kobayashi *et al.*, (2010), found an optimal pH value of 8.0 for a exo-polygalacturonase produced by the *Bacillus sp*. strain KSM-P576, while Devi and Rao, (1996), found an optimal pH of 10 for the polygalacturonase produced by the *Bacillus* sp. MG-cp-2.

3.3 Effect of some metal ions

The activity of the polygalacturonase secreted by *P. brevicompactum* was stimulated by Mg^{+2} ions. In the presence of this ion, a 160% increase in enzyme activity could be observed. The Cs^{+2}, Hg^{+2}, Li^{+2} and Sr^{+2} ions, on the other hand, inhibited enzyme activity. The ions Zn^{+2} and Cu^{+2} inhibited it by 94% and 69%, respectively.

Mg^{+2} also stimulated the activity of polygalacturonase produced by *Aspergillus carbonarius* (Devi and Rao, 1996), but inhibited the activity of polygalacturonase produced by *Bacillus* sp. (Kelly and Fogarty, 1978).

Tomazic, (1991) and Kobayashi; Koike and Yoshimatsu, (2010), highlight that the stabilization of some enzymes can be induced by non-protein additives, especially divalent ions such as Ca^{+2}, Mn^{+2}, Zn^{+2} and Mg^{+2}. If used in low concentrations, these ions can support the tertiary structure of the protein, promoting the formation of cross-links that provide a greater stability to it.

IV. CONCLUSION

After obtaining the results, one can conclude that the endophytic strain of *Penicilium brevicompactum* was able to use a wide range of carbon sources, but with polygalacturonase activity varying with each source. The highest yield, however, was achieved after 30 hours of incubation in the presence of 4% of galactose and 2% of pectin.

The characterization of the polygalacturonase revealed that the optimal temperature of this enzyme is 72 °C and that it maintains 60 and 15% of its maximum activity when incubated for 2 hours at 40 and 90°C, respectively.

The optimal pH for the activity of the enzyme was 4.6. The enzyme retained 65 and 30% of its maximum activity when incubated at pH 3.5 and 9.5, respectively, for 24 hours at ambient temperature.

The enzyme activity was stimulated by the Mg^{2+} ions. On the other hand, it was inhibited by the ions Cs^{+2}, Hg^{+2}, Li^{+2} and Sr^{+2}. The ions Zn^{+2} and Cu^{+2} inhibited it by 94% and 69%, respectively.

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